430 Rec'd PCT/PTO 02 JUL 1999

FORM PTO-1390 (Modified) (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
960-219US	09/341079

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US98/00246	02.01.98	02.01.97

TITLE OF INVENTION

STABILIZATION OF TRIPLEXES BY WATER STRUCTURE-MAKING SUBSTANCES

APPLICANT(S) FOR DO/EO/US

Jacques R. Fresco; John Laurence Richard Lavelle

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
    A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☒ A ~~change~~ new of power of attorney and/or address letter.
18. ☒ Certificate of Mailing by Express Mail
19. ☒ Other items or information:

    Express Mail Certificate EL145986440US
    Postcard

PCTUS1/REV03 (October 1997)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR) 09/341079	INTERNATIONAL APPLICATION NO. PCT/US98/00246	09Rec'd PCT/PTO 02 JUL 1999 ATTORNEY'S DOCKET NUMBER 960-219US

20. The following fees are submitted:.		CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Search Report has been prepared by the EPO or JPO	**$930.00**	
☒ International preliminary examination fee paid to USPTO (37 CFR 1.482)	**$720.00**	
☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))	**$790.00**	
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO	**$1,070.00**	
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)	**$98.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$720.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	32 - 20 =	12	x $22.00	**$264.00**
Independent claims	2 - 3 =	0	x $82.00	**$0.00**
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**

TOTAL OF ABOVE CALCULATIONS =		**$984.00**
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable)**.	☒	**$492.00**
SUBTOTAL =		**$492.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +		**$0.00**
TOTAL NATIONAL FEE =		**$492.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).**	☐	**$0.00**
TOTAL FEES ENCLOSED =		**$492.00**
	Amount to be: refunded	$
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☒ A check in the amount of **$492.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **23-3040** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Princeton, New Jersey 08542-0592

SIGNATURE

Richard C. Woodbridge
NAME

26,423
REGISTRATION NUMBER

July 2, 1999
DATE

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27 (d)) - NONPROFIT ORGANIZATION	Docket No. 960-219US

Serial No.	Filing Date	Patent No.	Issue Date
	Herewith		

Applicant/ Patentee: Jacques R. Fresco; John Laurence Richard Lavelle

Invention: STABILIZATION OF TRIPLEXES BY WATER STRUCTURE-MAKING SUBSTANCES

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: Princeton University

ADDRESS OF ORGANIZATION: 5 New South Building
Princeton, N.J. 08544-0036

TYPE OF NONPROFIT ORGANIZATION:

- ☒ University or other Institute of Higher Education
- ☐ Tax Exempt under Internal Revenue Service Code (26 U.S.C. 501(a) and 501(c)(3))
- ☐ Nonprofit Scientific or Educational under Statute of State of The United States of America
  Name of State: Citation of Statute:
- ☐ Would Qualify as Tax Exempt under Internal Revenue Service Code (26 U.S.C. 501(a) and 501(c)(3)) if Located in The United States of America
- ☐ Would Qualify as Nonprofit Scientific or Educational under Statute of State of The United States of America if Located in The United States of America
  Name of State: Citation of Statute:

I hereby declare that the above-identified nonprofit organization qualifies as a nonprofit organization as defined in 37 C.F.R. 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in:

- ☒ the specification to be filed herewith.
- ☐ the application identified above.
- ☐ the patent identified above.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the above-identified nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

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Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

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FULL NAME _____

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Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Allen J. Sinisgalli

TITLE IN ORGANIZATION: Associate Provost

ADDRESS OF PERSON SIGNING: Princeton University, 8 New South Building
Princeton, N.J. 08544-0036

SIGNATURE: Allen J. Sinisgalli DATE: 7/1/99

09/341079
PCT/US98/00246
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TITLE OF THE INVENTION

STABILIZATION OF TRIPLEXES BY WATER STRUCTURE-MAKING SUBSTANCES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of application Serial No. 60/034,592 filed January 2, 1997.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

The United States Government may have certain rights in this invention by virtue of NIH Grant GM42936 and NIH Biophysics Training Grant GM08309.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates to methods for stabilizing nucleic acid triplexes.

2. Description of Related Art

Oligonucleotide third strands can bind to double-stranded nucleic acids to form triple-stranded helices (triplexes) in a sequence specific manner. The third strand binding code (a complementarity principle) dictates the sequence specificity for binding third strands in the major groove of double-stranded nucleic acids to form a triple-stranded helix or triplex. The code provides the specificity of third-strand binding for design of gene-based therapeutic agents that bind specifically to target nucleic acid sequences with little or no non-specific binding to non-target sequences. The third strand binding code, as well as various utilities for triplexes, are described in United States Patents 5,422,251 and 5,693,471 to Fresco, which also shows ionic conditions such as the

presence of Mg^{+2}, Mn^{+2}, Ca^{+2}, Na^{+}, Li^{+}, K^{+} or tetramethylammonium cations suitable for triplex formation.

SUMMARY OF THE INVENTION

The present invention relates to methods for enhancing the stability of a triplex formed from one or more nucleic acid strands in a solution, said method comprising adding to the solution, either before or after formation of the triplex, an effective amount of either of the following:

(a) a water structure-making substance other than an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine; or

(b) a combination of said water structure-making substance and an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine.

The present invention further relates to a method for forming a triplex from one or more nucleic acid strands, said method comprising adding to a solution, in any order, the strand(s) and an effective amount of one of the following:

(a) a water structure-making substance other than an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine; or

(b) a combination of said water structure-making substance and an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine;

and allowing said triplex to form.

DETAILED DESCRIPTION OF THE INVENTION

As mentioned above, it has been discovered that water structure-making substances can stabilize triplexes in solution. By water structure-making substance is meant a substance which, when dissolved in water, will yield ions or other structures which interact with water more strongly than bulk water molecules with each other.

The water structure-making substances include organic cations, cationic lipids, organic anions, inorganic anions,

and water-miscible organic solvents. Preferred organic cations include alkylammonium (e.g., methylammonium, dimethylammonium, trimethylammonium, tetramethylammonium, and tetraethylammonium, triethylammonium, and their derivatives). Preferred cationic lipids include cetyltrimethylammonium, tridodecylmethylammonium, and 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanammonium and their derivatives. Preferred organic anions include acetate and its derivatives. Preferred inorganic anions include phosphate, sulfate, *etc.*, termed kosmotropes below. Preferred organic solvents include DMSO and alcohols, most preferably methanol, ethanol, 2-propanol, isopropanol and their derivatives.

The oligonucleotide third strand is a synthetic or natural oligonucleotide capable of binding with specificity to a predetermined target region of a double-stranded native nucleic acid molecule to form a triple-stranded structure. The third strand may bind solely to one strand of the native nucleic acid molecule, or may bind to both strands at different points along its length. The third strand need not be perfectly complementary to the duplex, but may be substantially complementary. In general, by substantially complementary is meant that one mismatch is tolerable in about every 10 base pairs.

The oligonucleotide may have a native phosphodiester backbone or may be comprised of other backbone chemical groups or mixtures of chemical groups which do not prevent a triple-stranded helix from forming. These alternative chemical groups include phosphorothioates, methylphosphonates, peptide nucleic acids (PNAs), and others known to those skilled in the art. Preferably, the oligonucleotide backbone is phosphodiester.

The oligonucleotide may also comprise one or more modified sugars, which would be known to those skilled in the art. As an example, such a sugar can be an α-

enantiomer.

The third strand may also incorporate one or more unnatural (for nucleic acids) heterocycle base substitutes if such is necessary or desirable to improve third strand binding. Examples of such unnatural heterocycle design and the heterocycles so designed are found in the co-pending U.S. application of Fresco, *et al.* entitled "Residues for Binding Third Strands to Complementary Nucleic Acid Duplexes of any Base-Pair Sequence", S.N. 08/473,888 filed June 7, 1995, the contents of which are incorporated herein by reference.

The third strand may also contain one or more of a variety of other substituents which can strengthen third strand binding to the target duplex. These include intercalators, crosslinkers, peptides, oligosaccharides, and their analogs and/or derivatives

While the triplex is preferably formed from three discrete strands (two strands which form the duplex target via Watson-Crick binding, and a third strand probe), the present invention also encompasses stabilization of triplexes formed from less than three discrete strands. For example, the triplex may be formed from a single stranded target, and a probe strand that has a sequence complementary to the target strand to form the target duplex, as well as a sequence at a different position which will bind to the formed duplex as if it were a third strand. Further, the triplex may be formed from a target duplex which comprises a single strand which hybridizes to itself via a hairpin turn, and a third strand probe. The triplex may also be formed from a single strand which forms a triplex by virtue of two hairpin turns.

The order of addition of the components of the invention is not critical. For example, the water structure making substance(s) may be added to a solution which already contains the triplex to be stabilized, or may be added along with one or more strands. Moreover, the water structure-making substance may be covalently linked

to the third strand in a manner which would be readily apparent to one of ordinary skill.

The term "solution" as used herein is intended to include both *in vitro* and *in vivo* environments. When dealing with *in vivo* solutions (*i.e.*, in a cell), it will be recognized that toxicity concerns will affect the nature and concentration of water structure making substances that can be employed. In general, cationic lipids will be preferred when dealing with *in vivo* solutions, and may be formulated with the third strand for cellular uptake in a manner known to those of ordinary skill.

The optimum concentration of water structure-making substance to be added may readily be determined by one of ordinary skill. Appropriate concentrations for many substances are set forth in the examples and tables *infra*.

While not wishing to be bound by any particular theory of how the present invention works, it is known that when a salt is dissolved in water, different anions and cations are observed to decrease, increase or have little effect on the volume of the solution. These alternative effects have been explained in terms of the interaction of the anion or cation with water molecules according to what is often called the multilayer hydration model. Briefly, this model of ion-water interaction divides the volume of an ion in solution, V_{ion}, into four components:

$$V_{ion} = V_{cryst} + V_{elect} + V_{disord} + V_{caged}$$

where: V_{cryst} is the volume of the ion based on its crystal radius; V_{elect} is the electrostriction volume (stronger ion-H_2O interaction decreases volume); V_{disord} is the disordered or void-space volume (weaker ion-H_2O interaction increases volume); and V_{caged} is the caged or structured volume (that occurs when a hydrophobic ion (organic cation) interacts with H_2O molecules, which decreases volume).

Although these ion volume factors are interdependent, the observed solution volume changes on addition of ions is

readily explained by this descriptive model. Using this model, ions can be divided into three classes: 1) electrostrictive "structure-making" ions when V_{elect} is dominant; 2) disordered "structure-breaking" ions when V_{disord} is dominant; and 3) hydrophobic "structure-making" ions when V_{caged} is dominant.

The volumes, V_{elect}, V_{disord} and V_{caged} have been calculated for a number of anions and cations (see Horne, R.A. (Ed.)(1972) *Water and Aqueous Solutions*, Wiley-Interscience, NY). As these volumes are additive, predictions of the solution volume effect of a particular salt can be made. The structure-making or structure-breaking tendency of anions based upon this model follows the rank order of the Hofmeister series, which is the relative tendency of anions to stabilize and solubilize proteins. A partial rank order is:

(destabilize proteins) ClO_4^- < Cl^- < CH_3COO^-< HPO_4^{2-} < SO_4^{2-} (stabilize proteins)

This rank order is also known as the "chaotropic series", as studies have shown Cl^- to have little effect on water-structure, whereas anions to the left of Cl^- are water structure-breakers (V_{disord} is dominant) called chaotropes (from the Greek, meaning disorder (*chao*)) because they destabilize proteins, while anions to the right of Cl^- are water structure-makers (V_{elect} is dominant) called kosmotropes (from the Greek, meaning order (*kosmos*)) because they stabilize proteins. Thus, polar or charged chaotropes "disrupt" the structure of water because they interact with water less strongly, while polar or charged kosmotropes interact with water more strongly than bulk water molecules with each other.

Previous work has shown that the effect of various salts on the stability of duplex DNA also follows the Hofmeister series (Hamaguchi and Geiduschek, *JACS* 84 (8),

1329-38 (1962)). In the same study it was concluded that at the very high concentrations needed to observe the anion effects, there were only minor differences observed when the cations Li^+, Na^+, K^+, and TMA^+ were varied.

The results obtained in accordance with the present invention show that the effect of anions on triplex stability follows the Hofmeister series. For the triplex $d(C^+-T)_6$:$d(A-G)_6$.$d(C-T)_6$ in 2.0 M anion at pH 7.0, rank according to triplex Tm values (°C) for the various salts is:

$NaClO_4$ (inhibits triplex formation) < NaCl (7°) < $NaOOCCH_3$ (15°); Na_2HPO_4 (15°) < Na_2SO_4 (21°) < $(NH_4)_2SO_4$ (28°).

For the triplex $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$ in 2.0 M anion at pH 7.0, rank according to triplex Tm values (°C) for the various salts is:

$NaClO_4$ (44°) < NaCl (66°); $NaOOCCH_3$ (66°) < Na_2HPO_4 (80°); Na_2SO_4 (80°) < $(NH_4)_2SO_4$ (83°).

Whereas duplex DNA stability is not greatly affected by cations in general when they are at very high concentration, the applicants have found that organic cations have a strong effect on triplex stability. Their stabilizing ability can also be explained by the ion-water model. Thus, for these organic cations V_{caged} is dominant, and in this case water "structure-making" occurs as a result of the hydrophobic cation. That is, the organic cation (kosmotrope) interacts much less strongly with water, and in so doing orders the water molecules around them (the effect on the interfacial water surrounding the nonpolar substance is that it becomes more ordered).

For the triplex $d(C^+-T)_6$:$d(A-G)_6$•$d(C-T)_6$ at pH 7.0:

TPA-Cl (inhibits triplex formation) < NaCl < MA-Cl < DMA-Cl < TEA-Cl < TMA-Cl < TriMA-Cl.

For the triplex $d(T)_{21}$:$d(A)_{21}$•$d(T)_{21}$ at pH 7.0, the highest obtainable Tm is 72 °C in 5.0 M NaCl, while the

highest obtainable Tm is 95 °C in 6.0 M TMA-Cl.

As both triplexes and duplexes have a high negative charge density, they are stabilized in turn by cations of positive charge density. Therefore, although V_{caged} is negative for the organic cations, TMA^+ (-21 cm^3 $mole^{-1}$), TEA^+ (-18 cm^3 $mole^{-1}$), and TPA^+ (-24 cm^3 $mole^{-1}$), (because of their water structure-making nature, *i.e.*, decreased volume), TPA^+ must not have sufficient positive charge density. Thus, it is likely that the size (and hence charge density) of these organic cations also plays a role in their tendency to stabilize triplexes. At pH 7.0 they all have one positive charge and therefore their charge density will scale with their surface area (calculated using ChemPlus in HyperChem (HyperChem 4.0 (1994) Hypercube Corp., Waterloo, Ontario, Canada)): MA^+ (178 Å^2), DMA^+ (208 Å^2), $TriMA^+$ (232 Å^2) TMA^+ (252 Å^2), TEA^+ (325 Å^2), and TPA^+ (383 Å^2). This implies that $TriMA^+$ and TMA^+ have the optimum size and charge density to stabilize triplexes with homopyrimidine third strands. However, as observed, their decreasing charge density also makes them less soluble in H_2O, and this may also have an effect.

It should be noted that TEA^+ and TPA^+ have a significant destabilizing effect on the duplex $d(A\text{-}G)_6.d(C\text{-}T)_6$.

Triplexes are stabilized by certain alcohols, PEG, and DMSO as follows.

For the triplex $d(C^+\text{-}T)_6:d(A\text{-}G)_6 \bullet d(C\text{-}T)_6$ at pH 7.0:

MeOH < EtOH < 2-PrOH < 1-BuOH.

For the triplex $d(T)_{21}:d(A)_{21}.d(T)_{21}$ in 50 Vol% alcohol + MB (0.15 M Na^+/0.005 M Mg^{++}/0.01 M cacodylate titrated to the desired pH) at pH 7.0, the rank order based on Tm values (°C) is:

0%, MB only (23°) < MeOH (38°) < EtOH (53°) < 2-PrOH (65°).

For the triplex poly r(U:A•U) in Vol% EtOH + 0.016 M NaCl at pH 7.0:

0% (26°) < 10% (39°) < 20% (42°) < 30% (45°) < 50% (53°).

For the triplex $d(C^+-T)_6:d(A-G)_6 \cdot d(C-T)_6$ in 20 Vol% PEG(ave. molecular weight) + MB at pH 7.0:

0%, MB only (11°) < PEG200 (18°) < PEG400 (22°) < PEG600 (24°).

For the triplex $d(C^+-T)_6:d(A-G)_6 \cdot d(C-T)_6$ in Vol% DMSO + MB at pH 7:

0%, MB only (11°) < 10% (15°) < 20% (17°) < 40% (20°) < 50% (27°) < 60% (15°).

For the triplex $d(T)_{21}:d(A)_{21} \cdot d(T)_{21}$ in Vol% DMSO + MB at pH 7.0:

0%, MB only (23°) < 30% (34°) < 40% (38°) < 50% (15°).

Water-miscible neutral polar organic substances can also be classified as water structure-breaking (chaotropes) or water structure-making (kosmotropes) (see Collins and Washabaugh, *Q.Rev.Biophysics* (18) 323-422 (1985)). The low molecular weight alcohols are water structure-making, as is the neutral hydrophilic polymer PEG and the potent H-bond acceptor DMSO.

It would appear therefore that substances that are water structure-making enhance the stability of triplexes. Conversely, no water structure-breaking substance has been observed to enhance triplex stability. This thermodynamic model of ion-water interaction has given a thermodynamic answer. We now attempt to relate this thermodynamic understanding of how "altered water structure" may influence the conformation of DNA to the molecular mechanism for triplex formation.

The result of water-alcohol, water-PEG, or water-DMSO interaction is that it reduces the water available to hydrate other 'solutes'. This is a well known observation for DNA in water/ethanol mixtures. The higher the proportion of ethanol, the less the proportion of water available for hydration of DNA (i.e., dehydration), and in 60 to 70 % ethanol there is sufficient dehydration to induce a conformational change in DNA from B to A or Z.

Such conformational changes require varying degrees of unwinding the DNA, with resultant changes in rotation of the nucleotide residues from 36 - 45 ° to 30 - 33 ° in the case of B to A, and even *anti* to *syn* isomerization in the case of B to Z.

In this connection, the unwinding of duplex DNA increases in the presence of MeOH, EtOH, ethylene glycol and DMSO but not glycerol (Lee, et al., (1981) *Proc.Natl.Acad.Sci.U.S.A.* **78**, 2838-2842). Moreover, the degree of unwinding is a continuous process in response to the concentration of organic solvent. The Vol % of organic solvent required for unwinding increases in the order: DMSO < MeOH < EtOH < ethylene glycol.

As glycerol does not enhance triplex stability, it is likely that MeOH, EtOH, 2-PrOH, PEG and DMSO all enhance triplex stability by facilitating unwinding of the duplex. In fact, it would make sense that all compounds that facilitate both a B to A/Z transition and dehydration also enhance binding of third strands that must enter the major groove of the duplex. Clearly, third strand binding must require displacement of water from the major groove of the duplex to accommodate this extra strand. This is further supported by the observation that RecA facilitates third strand binding, since the hydrophobic environment created by the protein must facilitate removal of water (Iyer, *et al.*, *J.Biol.Chem.* 270, 14712-717 (1995)).

Thus, it appears that substances that are water structure-making enhance the stability of triplexes. They may do so by two associated mechanisms: by facilitating the unwinding of the duplex to the extent needed to accommodate the third strand, which need not necessarily involve a B to A transition, and by facilitating the removal of water from the major groove to permit third strand binding.

EXAMPLES

To illustrate the various aspects and processes of the

invention, the effects of different additives on the stability of three different triplexes is documented below. It is understood that these examples are not intended to limit the scope of the invention and that other embodiments of the invention will be apparent from the information provided to those of ordinary skill in the art.

Example 1

$d(C^+-T)_6:[d(A-G)_6 \cdot d(C-T)_6]$

$d(A-G)_6$ and $d(C-T)_6$ were synthesized, purified and analyzed as described in Lavelle and Fresco, *Nucleic Acids Res.* 23, No. 14, 2692-2705 (1995). Briefly, the strands were synthesized using standard phosphoramidite chemistry on an Applied Biosystems 380B synthesizer. The oligomers were purified by reverse phase HPLC (0.1 M triethylammonium acetate pH 7.0/acetonitrile) and ion exchange HPLC (5 M urea/20 mM sodium phosphate pH 6.0, 5 M urea/20 mM sodium phosphate/1 M sodium sulfate pH 6.0) and desalted by reverse phase chromatography using C18 Sep-Pak. Molar extinction coefficients determined after phosphodiesterase I digestion, ε_{260} = 9890 for $d(A-G)_6$ and ε_{260} = 8510 for $d(C-T)_6$ at 25 °C in 2.6 x 10^{-5} M Tris pH 7.4 / 2.4 x 10^{-5} M $MgCl_2$, were used to determine oligomer concentration. The triplex mixture was made with equimolar stocks of the two strands; after forming the duplex, a stoichiometric amount of the third strand was added (which is the same as the homopyrimidine strand of the core duplex in this case).

Absorption spectra and thermal melting profiles were determined in a computer driven AVIV 14DS spectrophotometer equipped with a thermoelectrically controlled cell holder for cells of 1 cm pathlength. Filtered, dry air was passed through the cell compartment to prevent condensation on the cell walls at low temperatures. The flow rate was set low enough so as not to create a temperature gradient between the sample and the cell holder, which was confirmed by

monitoring the temperature in the sample and cell holder during trial melting profiles. For melting experiments, spectra were measured every 1 nm and 2 °C. Only triplex and duplex transitions that occur between 0 and 100 °C were observed. Care was taken to obtain true equilibrium melting profiles by recording scans only after a cuvette was allowed to reach the desired temperature (8 min). This ensured that the rate of temperature rise is less than the rate of the association-dissociation reaction under study, as confirmed by the absence of further absorbance change on longer incubation at some fixed temperature within the transition. These spectra were used to obtain melting profiles and their derivatives at appropriate wavelengths, from which melting transition temperatures, Tm values, were obtained from the midpoint of the transition. Tm values ($T_m \pm 0.5$ °C) were obtained by measuring each melting profile at least twice. Unless otherwise stated, Tm and % hypochromicity values were obtained from melting profiles at 260 nm. All UV-melting profiles, wavelength scans and difference spectra are plotted using raw data. % Hypochromicity was calculated using:

$$\frac{A_{260}(\text{duplex + coil}) - A_{260}(\text{triplex})}{A_{260}(\text{duplex + coil})} \times 100$$

for 3→2 + 1 transitions; and

$$\frac{A_{260}(\text{coil}) - A_{260}(\text{triplex})}{A_{260}(\text{coil})} \times 100$$

for 3→1 + 1 + 1 transitions.

The effect of various additives on triplex stability was determined, and the results are presented below in Tables 1-1 to 1-28. These results were obtained at pH 7 unless otherwise noted. The abbreviation "MB" denotes a mixing buffer comprised of 0.15 M NaCl, 0.005 M $MgCl_2$ and

0.01 M cacodylate, titrated to the desired pH.

Table 1-1--Methylammonium chloride (MA-Cl)

Molarity	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
1.0	18	13	53	8
2.0	19	12	53	8
3.0	20	13	52	6
4.0	19	11	51	8

Table 1-2--Dimethylammonium chloride (DMA-Cl)

Molarity	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
1.0	20	11	53	8
2.0	23	10	52	8
3.0	26	9	51	6
4.0	27	10	49	8

Table 1-3--Trimethylammonium chloride (TriMA-Cl)

Molarity	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
1.0	28	10	52	6
2.0	36	9	52	7
3.0 (pH 3.7)	72(a)	19	--	--
3.0 (pH 4.9)	67(a)	15	--	--
3.0 (pH 5.8)	58(a)	15	--	--
3.0	50(a)	17	--	--
3.0 M (pH 7.4)	36	8	51	7
3.0 M (pH 7.8)	--	--	52	8
4.0 M	53(a)	16	--	--
4.0 M (pH 7.4)	36	8	50	6

(a) Tm for a 3→1 transition.

Table 1-4--Tetramethylammonium chloride (TMA-Cl)

Molarity		1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
1.0		31	10	54	9
2.0		29	9	55	7
3.0	(pH 3.7)	75(a)	18	--	--
3.0	(pH 4.9)	72(a)	16	--	--
3.0	(pH 5.8)	61(a)	14	--	--
3.0		30	10	56	9
4.0		43	9	59	7
6.0		50(a)	23	--	--
6.0	(pH 6.0)	67(a)	23	--	--

(a) Tm for a 3→1 transition.

Table 1-5--Tetraethylammonium chloride (TEA-Cl)

Molarity	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
0.5	16	10	43	6
1.0	22	13	43	10
1.6	26	9	37	7
2.0	insoluble			

Table 1-6--Tetrapropylammonium chloride (TPA-Cl)

Molarity		1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
0.1		--	--	29	8
0.5		--	--	31	8
0.9		--	--	28	8
0.9	(pH 8.5)	--	--	27	8
1.0		insoluble			

Table 1-7--Cetyltrimethylammonium chloride (CTriMA-Cl)

Wt %	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
10^{-4}	--	--	20	10
10^{-3}	--	--	23(a)	5
10^{-2}	28(b)	5	--	--
10^{-1}	insoluble, micelle formation			
10^{-4} + MB	10	11	50	13
10^{-3} + MB	22(c)	8	52	14
10^{-2} + MB	insoluble, micelle formation			
10^{-3} + MB + 0.02 M TMA	12	8	51	10
10^{-3} + MB + 0.1 M TMA	14	11	51	9
10^{-3} + MB + 0.2 M TMA	15	10	52	8
10^{-3} + MB + 0.4 M TMA	16	11	52	8
10^{-2} + MB + 0.1 M TMA	45	11	64	11
10^{-2} + MB + 0.2 M TMA	insoluble, micelle formation			

(a) Tm for duplex melting.

(b) Tm 3→2+1 transition; phase transition of CTriMA masks duplex transition.

(c) very broad transition (3-40°C).

Table 1-8--Tridodecylmethylammonium chloride (Tridodecyl MA-Cl)

Wt%	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
10^{-4}	--	--	20	10
10^{-3}	--	--	20	10
10^{-3} (f)	41(d)	14	--	--
10^{-2}	insoluble, micelle formation			
10^{-4} + MB	10	10	51	11
10^{-3} + MB	11	9	51	10
10^{-2} + MB	insoluble, micelle formation			

(d) Tm for a 3→1 transition.

(f) pH 6.0.

Table 1-9--2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA)

Wt%	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
10^{-4}	--	--	21	11
10^{-4} (f)	40(d)	10	--	--
10^{-3}	22(d)	7	--	--
10^{-3} (f)	40	6	79	7
10^{-2}	27	6	77	25(e)
10^{-1}	insoluble, micelle formation			
10^{-4} + MB	10	11	50	16
10^{-3} + MB	12	7	50	15
10^{-2} + MB	insoluble, micelle formation			

(d) Tm for a 3→1 transition.

(e) significant overlap with phase transition of DOSPA.

(f) pH 6.0.

Table 1-10--Sodium Dodecylsulfate (SDS)

Wt%	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.1 + MB	--(a)	--	51	12
1 + MB	--(a)	--	51	11
10 + MB	--(a)	--	54	12

(a) appears to inhibit triplex formation; however, any transitions below 20°C are not observable as the solution solidifies ≤20°C.

Table 1-11--Tetramethylammonium Sulfate (TMA-S)

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.1	16	7	48	21
0.5	20	17	56	11
1.0	25	14	57	11
1.5	TMA-S precipitates			

Table 1-12--Trehalose

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.75 + MB	12	7	47	10
1.5 + MB	12	4	44	10
2.0 + MB	13	4	41	9

Table 1-13--Glycerol

Vol %	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
10 + MB	11	9	49	10
20 + MB	12	9	45	10
30 + MB	12	6	42	12
30 + MB + 1.0 M TriMA	19	8	45	9

Table 1-14--Poly(ethylene glycol) (PEG)

Vol %/(MW)	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
20 (200) + MB	18	11	44	10
40 (200) + MB	33(b)	23	--	--
20 (400) + MB	22	22	48	17
20 (600) + MB	24	15	49	14

(b) Tm for a 3→1 transition.

Table 1-15--Dimethyl Sulfoxide (DMSO)

Vol %	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
10 + MB	15	12	48	12
20 + MB	17	11	45	12
40 + MB	20	12	41	12
50 + MB	27(b)	17	--	--
60 + MB	15(b)	12	--	--
60 + MB(f)	36(b)	24	--	--

(b) Tm for a 3→1 transition.

(f) pH 6.0.

Table 1-16--Mixing Buffer (0.15M NaCl; 0.005M $MgCl_2$)

pH	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
4.2	32	9	62	13
5.0	29	12	50	9
7.0	11	12	50	10
7.5	1	4	50	10

Table 1-17--NaCl

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	7	7	53	12
0.5	8	14	54	10
0.8	10	16	56	11
0.9	10	17	56	11
1.0	9	17	54	12
2.0	7	13	54	12
3.0	5	2	57	7
5.0	--	--	51	10
6.0	NaCl crystallizes			

Table 1-18--Na_2HPO_4

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	8	9	54	12
0.8	12	11	56	11
2.0	15	11	57	11
2.0 (pH 6.5)	29	14	59	11
3.0	Na_2HPO_4 crystallizes			

Table 1-19--Sodium Acetate

Molarity	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
0.4	9	12	53	11
0.8	12	12	56	11
2.0	15	13	55	11
3.0	16	13	54	12

Table 1-20--Sodium Sulfate

Molarity	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
0.4	14	12	54	10
0.8 (pH 7.2)	9	8	56	11
0.8	17	13	55	12
2.0	21	14	58	11
3.0	crystallizes			

Table 1-21--Sodium Perchlorate

Molarity	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
2.0	--	--	42	11
2.0 (pH 6.0)	18	12	43	10

Table 1-22--Ammonium Chloride

Molarity	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
0.4	8	5	56	12
0.8	--	--	54	12
2.0	--	--	58	12

Table 1-23--Ammonium Sulfate

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	8	4	55	12
0.8	19	13	57	12
2.0	28	13	58	11
3.0	37(b)	14	60(b)	10

(b) overlapping transitions.

Table 1-24--Methanol (MeOH)

% MeOH	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
10% + MB	15	13	51	11
20% + MB	15	14	48	11
30% + MB	15	12	44	12
60% + MB	16	7	37	7
70% + MB	16	7	35	7
80% + MB	no transitions observed			

Table 1-25--Ethanol (EtOH)

% EtOH	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
10% + MB	12	12	48	10
20% + MB	13	12	44	11
30% + MB	15	6	41	11
40% + MB	15	7	36	10
50% + MB	21	23	38	8
60% + MB	40(a),(c)	31	--	--
70% + MB	no transitions observed			
50% + 1.5 M TriMA	30(c)	16	--	--

(a) broad transition (20-60°C).

(c) Tm for a 3→1 transition.

Table 1-26--2-Propanol (2-PrOH)

% Propanol	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
5% + MB	9	7	49	7
10% + MB	11	14	47	12
20% + MB	17	9	43	13
30% + MB	20	11	40	12
40% + MB	27(b)	31	39(b)	15
50% + MB	40(c)	38	--	--
60% + MB	insoluble			
30% + 20% EtOH + 3 M TMA	32(c)	10	--	--
40% + 3 M TMA	phase separation			
50% + 3 M TMA	insoluble			

(b) overlapping transitions.

(c) Tm for a 3→1 transition.

Table 1-27--1-Butanol (BuOH)

% BuOH	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.1% + MB	8	10	51	9
1% + MB	7	9	50	9
5% + MB	7	10	47	9
10% + MB	phase separation			

Example 2

$d(T)_{21}$:[$d(A)_{21}$•$d(T)_{21}$]

Triplexes were formed and tested as in Example 1, except that the strands $d(T)_{21}$ and $d(A)_{21}$ were used instead of $d(A-G)_6$ and $d(C-T)_6$. The concentrations of these strands were calculated using the molar extinction

coefficients for poly (dA) (ε_{257}=8600) and for poly (dT) (ε_{265}=8700) at 25 °C. The results are shown below in Tables 2-1 to 2-10.

Table 2-1--NaCl

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.4	24	17	58	19
0.8	42	18	62	17
1.0	49(a)	18	64(a)	18
2.0	66(b)	33	--	--
3.0	70(b)	33	--	--
5.0	72(b)	33	--	--
6.0	NaCl crystallizes			

(a) overlapping transitions.
(b) Tm for a 3→1 transition.

Table 2-2--Ammonium Chloride

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
1.0	65	36	--	--
2.0	71	36	--	--
3.0	76	36	--	--

Table 2-3--Ammonium Sulfate

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
1.0	71	36	--	--
2.0	83	36	--	--
3.0	93	36(c)	--	--

(c) obtained by extrapolation.

Table 2-4--Trimethylammonium chloride (TriMA-Cl)

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
1.0 + MB	45	16	61	11
2.0 + MB	66(b)	27	--	--
3.0 + MB	70(b)	26	--	--
1.0	39	17	63	18

(b) Tm for a 3→1 transition.

Table 2-5--Tetramethylammonium salts (TMA)

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
1.0 TMA-Chloride	28	17	65	19
6.0 TMA-Chloride	95(b)	33(c)	--	--
1.0 TMA-Sulfate	54	10	74	13
1.5 TMA-Sulfate	TMA-S precipitates			

(b) Tm for a 3→1 transition.

(c) obtained by extrapolation.

Table 2-6--Sodium salts (all 2.0 M)

Salt Added	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
Na_2HPO_4	80(b)	33	--	--
$NaOOCCH_3$	66(b)	33	--	--
Na_2SO_4	80(b)	33	--	--
$NaClO_4$	44(b)	30	--	--

(b) Tm for a 3→1 transition.

Table 2-7--Alcohols (all 50 Vol%)

Alcohol Added	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
Methanol + MB	38(a)	38	--	--
Ethanol + MB	53(a)	74	--	--
2-Propanol + MB	65(a)	74	--	--

(a) Tm for a 3→1 transition.

Table 2-8--Dimethyl Sulfoxide

Vol %	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
30 + MB	34(b)	18	44(b)	16
40 + MB	38(a)	35	--	--
50 + MB	15	9	34	28

(a) Tm for a 3→1 transition.

(b) overlapping transitions.

Table 2-9--Poly(ethylene glycol)

Vol % (MW)	1st Transition Tm °C	1st Transition Hypochromicity %	2nd Transition Tm °C	2nd Transition Hypochromicity %
20 (200) + MB	39(a)	25	--	--
40 (200) + MB	41(a)	45	--	--
20 (600) + MB	52(a)	54	--	--

(a) Tm for a 3→1 transition.

Table 2-10--0.15 M NaCl+0.005 M $MgCl_2$

	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
	23	18	53	15

Example 3

Poly(rU):Poly(rA)•Poly(rU)

Triplexes were formed using the strands Poly(rU) and Poly(rA) (the same samples used in the work of Broitman, *et al.*, (1987) *Proc.Natl.Acad.Sci. U.S.A.* 84, 5120-5124). The results in Tables 3-1 to 3-4 were obtained by the standard UV melting protocols described in Example 1.

Table 3-1--Ethanol + 0.016 M NaCl

Vol % EtOH	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
10	39(a)	37	--	--
20	42(a)	39	--	--
30	45(a)	40	--	--
50	53(a)	54	--	--
60	insoluble			

(a) Tm for a 3→1 transition.

Table 3-2--Cetyltrimethylammonium chloride + 0.016M NaCl

Wt %	1st Transition		2nd Transition	
	Tm	Hypochromicity	Tm	Hypochromicity
	°C	%	°C	%
10^{-4}	27	16	40	20
10^{-3}	38(b)	12	63(b)	18
10^{-2}	insoluble, micelle formation			

(b) overlapping transitions.

Table 3-3--Trimethylammonium chloride + 0.016 M NaCl

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.020	34	21	42	18
0.053	44(a)	37	--	--
0.600	69(a)	41	--	--

(a) Tm for a 3→1 transition.

Table 3-4--Tetramethylammonium chloride + 0.016 M NaCl

Molarity	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
0.020	31	18	40	17

Table 3-5--0.16 M NaCl

	1st Transition		2nd Transition	
	Tm °C	Hypochromicity %	Tm °C	Hypochromicity %
	26	17	40	23

We claim:

1. A method for enhancing the stability of a triplex formed from one or more nucleic acid strands in a solution, said method comprising adding to the solution, either before or after formation of the triplex, an effective amount of either of the following:

(a) a water structure-making substance other than an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine; or

(b) a combination of said water structure-making substance and an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine.

2. The method of claim 1, wherein the water structure-making substance comprises an organic cation other than tetramethylammonium.

3. The method of claim 2, wherein the organic cation is selected from the group consisting of methylammonium, dimethylammonium, trimethylammonium, and tetraethylammonium.

4. The method of claim 1, wherein the water structure-making substance comprises a cationic lipid.

5. The method of claim 4, wherein the cationic lipid is selected from the group consisting of cetyltrimethylammonium, tridodecylmethylammonium, and 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanammonium.

6. The method of claim 1, wherein the water structure-making substance is selected from the group consisting of dimethyl sulfoxide and poly(ethylene glycol).

7. The method of claim 1, wherein the water structure-making substance comprises an organic anion.

8. The method of claim 7, wherein the organic anion is acetate.

9. The method of claim 1, wherein the water structure-making substance comprises an inorganic anion.

10. The method of claim 9, wherein the inorganic

anion is selected from the group consisting of phosphate, sulfate, cyanate, isocyanate and isothiocyanate.

11. The method of claim 1, wherein the water structure-making substance comprises a water-miscible organic solvent.

12. The method of claim 11, wherein the water structure-making substance comprises an alcohol.

13. The method of claim 12, wherein the alcohol is selected from the group consisting of methanol, ethanol, isopropanol and 2-propanol.

14. The method of claim 1, wherein the third strand comprises DNA or RNA.

15. The method of claim 1, wherein the third strand comprises an unnatural heterocycle base substitute, a base analog, an unnatural backbone, or a substituent which strengthens binding of the third strand in the triplex.

16. A method for forming a triplex from one or more nucleic acid strands, said method comprising adding to a solution, in any order, the strand(s) and an effective amount of one of the following:

(a) a water structure-making substance other than an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine; or

(b) a combination of said water structure-making substance and an alkali or alkaline earth metal cation, a tetramethylammonium cation, or a polyamine;

and allowing said triplex to form.

17. The method of claim 16, wherein the water structure-making substance comprises an organic cation other than tetramethylammonium.

18. The method of claim 17, wherein the organic cation is selected from the group consisting of methylammonium, dimethylammonium, trimethylammonium, and tetraethylammonium.

19. The method of claim 16, wherein the water structure-making substance comprises a cationic lipid.

20. The method of claim 19, wherein the cationic

lipid is selected from the group consisting of cetyltrimethylammonium, tridodecylmethylammonium, and 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanammonium.

21. The method of claim 16, wherein the water structure-making substance is selected from the group consisting of dimethyl sulfoxide and poly(ethylene glycol).

22. The method of claim 16, wherein the water structure-making substance comprises an organic anion.

23. The method of claim 22, wherein the organic anion is acetate.

24. The method of claim 16, wherein the water structure-making substance comprises an inorganic anion.

25. The method of claim 24, wherein the inorganic anion is selected from the group consisting of phosphate and sulfate.

26. The method of claim 16, wherein the water structure-making substance comprises a water-miscible organic solvent.

27. The method of claim 26, wherein the water structure-making substance comprises an alcohol.

28. The method of claim 27, wherein the alcohol is selected from the group consisting of methanol, ethanol, isopropanol and 2-propanol.

29. The method of claim 16, wherein the third strand comprises DNA or RNA.

30. The method of claim 16, wherein the third strand comprises an unnatural heterocycle base substitute, a base analog, an unnatural backbone, or a substituent which strengthens binding of the third strand in the triplex.

31. The method of claim 1, wherein the water structure-making substance is covalently linked to the third strand.

32. The method of claim 16, wherein the water structure-making substance is covalently linked to the third strand.

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Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

STABILIZATION OF TRIPLEXES BY WATER STRUCTURE MAKING SUBSTANCES

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on January 2, 1998 as United States Application No. or PCT International Application Number PCT/US98/00246

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.58.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

PRINCETON ORPA/DEVELOP TEL:609-258-1159 Jun 30'99 11:07 No.004 P.02

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

60/034,592	01/02/97
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may leopardize the validity of the application or any patent issued thereon.

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POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

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PRINCETON ORPA/DEVELOP TEL:609-258-1159 Jun 30'99 11:08 No.004 P.04

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